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Year: 2014

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## Identification of Synergistetes in endodontic infections

do Cabo Fernandes, Claudia ; Rechenberg, Dan-Krister ; Zehnder, Matthias ; Belibasakis, Georgios N

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DOI: <https://doi.org/10.1016/j.micpath.2014.05.001>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-96171>

Journal Article

Accepted Version

Originally published at:

do Cabo Fernandes, Claudia; Rechenberg, Dan-Krister; Zehnder, Matthias; Belibasakis, Georgios N (2014). Identification of Synergistetes in endodontic infections. *Microbial Pathogenesis*, 73:1-6.

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## Identification of *Synergistetes* in endodontic infections

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## **Abstract**

The bacterial phylum *Synergistetes* consists of Gram-negative anaerobes. Oral *Synergistetes* are divided in two main clusters, namely A and B. Increasing evidence demonstrates their involvement in etiology of oral infections, including apical periodontitis. This condition causes bone loss around the apex of the tooth, subsequent to pulp inflammation (pulpitis). Although the presence of *Synergistetes* has been confirmed in endodontic infections by molecular methods, these have not been morphologically identified in the affected apical region, and their prevalence among different endodontic infections has not been determined. Therefore, the aim of this study was to evaluate the prevalence, levels and morphology of oral *Synergistetes* clusters A and B, in apical root canal samples obtained of teeth with irreversible pulpitis, pulp necrosis and apical periodontitis, or previously root filled teeth with apical periodontitis. For their detection, fluorescence *in situ* hybridization and epifluorescence microscopy were used. *Synergistetes* cluster A was not detected in pulpitis, but was found in both apical periodontitis groups, more frequently and at higher ranges in teeth which were previously root filled. Microscopically, they appeared as straight or slightly curved long rods. *Synergistetes* cluster B was not detected in any of the cases. *Fusobacteria* and *Actinomyces*, which are well-established taxa in endodontic infections, were detected more frequently and at higher ranges than *Synergistetes*. In conclusion, *Synergistetes* cluster A constitutes part of the mixed apical microbiota in apical periodontitis, and may be involved in its pathogenesis.

**Keywords:** *Synergistetes*, apical periodontitis, pulpitis, apical, endodontic, oral bacteria

## 1. Introduction

*Synergistetes* is a recently identified bacterial phylum consisting of Gram-negative anaerobes. They are found in several microenvironments and constitute part of the human microbiota in health and disease [1-4], including the oral microbiota [5]. Phylogenetically, the oral *Synergistetes* are divided principally into clusters, namely cluster A and cluster B [2, 3]. Considerable work on the role of *Synergistetes* has been made in the field of periodontal infections. They are detected more frequently and more abundantly in subgingival plaque from periodontitis-affected than healthy sites [2], or in the saliva of patients with periodontitis, compared to healthy individuals [6]. Accordingly, dental plaque from periodontitis-affected sites exhibits higher clonal abundance and diversity of *Synergistetes*, in comparison to healthy sites [7]. The presence of *Synergistetes* in dental plaque is also more pronounced in necrotizing ulcerative gingivitis, compared to plaque-induced gingivitis [8].

Wide-spread oral infections, such as caries, periodontitis, and apical periodontitis, are of largely opportunistic nature [9]. Apical periodontitis (AP) is the outcome of endodontic infection. It is very prevalent among adults, with an estimated one-third of the population being affected [10]. The initial steps of the disease involve the microbial invasion and inflammation of the pulpal tissue (pulpitis), primarily as a result of dental caries [11, 12]. Persistent inflammation inside the root canal system causes degradation of the pulpal tissue [13] and allows for the progression of the endodontic infection. Histopathologically, this leads to the establishment of an inflammatory lesion in the bone around the apical region of the tooth, which is characteristic of AP, aiming to keep under control the recurring the infection in the root canal system [14].

*Synergistetes* have been frequently detected at elevated numbers in root canals of teeth with chronic endodontic infections, such as AP, as identified by polymerase chain reaction (PCR)-based methods and sequencing analyses [15, 16]. The size of the total *Synergistetes* population in necrotic teeth with AP is reported to range between  $10^4$  and  $10^6$  16S rRNA gene copies, while their proportion constitutes <1.0% of the total microbial community, allegedly within the detection range of other pathogens characteristic of endodontic infections [17]. Yet the differential presence of *Synergistetes* clusters A and B in the apical region of teeth with various clinical endodontic diagnoses has not been determined. Hence, the aim of this study was to evaluate the prevalence, levels and morphology of oral *Synergistetes* clusters A and B, in apical samples obtained of teeth with irreversible pulpitis but normal apical tissues, pulp necrotic teeth with AP, or previously root filled teeth with AP. Comparatively, the presence of *Fusobacteria* and *Actinomyces* was also evaluated, as these are well-established taxa in mixed endodontic infections.

## **2. Materials and Methods**

### ***2.1 Patients, procedures and sample collection***

This study was approved by the Ethics Review Board of the Canton of Zürich (KEK-ZH-No. 2011-0253/4) and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. The participating patients were in need of root canal treatment, and were treated at the Clinic for Preventive Dentistry, Periodontology and Cariology, University of Zürich, Center of Dental Medicine, Switzerland by one operator specialized in endodontics (DKR). The patients were asked if they were willing to participate in the study when they were of full age ( $\geq 18$ y), and were excluded from the study if they: i) were unwilling to

participate, ii) were under long-term anti-inflammatory medication, such as immunosuppressive chemotherapy or any antibiotic medication, iii) suffered from systemic illness (i.e. cardiovascular and respiratory disease, diabetes mellitus, HIV infection or hepatitis), or iv) were pregnant or in lactation. The clinical condition was diagnosed according to the patient's case history, clinical inspection, palpation, tenderness to percussion, vitality testing, probing depth and single-tooth radiographic examination. The clinical conditions included in the study were irreversible pulpitis but normal apical tissues (pulpitis, n=27), pulp necrotic teeth with apical periodontitis (N-AP, n=33), or apical periodontitis associated with a root-filled tooth (R-AP, n=21). Only one tooth per patient was included in the study. None of the sampled teeth exhibited a confirmed combined endodontic-periodontal lesion. All patients who entered the study gave written informed consent. All operative procedures were performed under a dental microscope and rubber dam isolation. The endodontic access was prepared with a sterile diamond-coated bur and the root canals were instrumented using ProTaper instruments (Maillefer Dentsply, Ballaigues, Switzerland), while endodontic working length was determined endometrically (Root ZX mini, J Morita Corp., Tustin, CA) with a hand file (Maillefer, Dentsply). The root canals were instrumented up to their apical constriction, under continuous manual irrigation with 1% NaOCl. During this process, a size-15 hand file was also used, to keep the apical foramen patent. After instrumentation to ProTaper F2, 5 ml of sterile physiological saline solution were administered to full working length to inactivate possible remnants of NaOCl. After drying the root canal with sterile paper points, a fine paper point (Orbis Dental, Münster, Germany) was inserted approximately 2 mm above the apical foramen to collect the apical tissue fluid, and the point was kept in

that position for 30 sec. Three consecutive paper points were collected from one canal and immediately after frozen at -80°C until further processing.

## ***2.2 Sample preparation***

Initially, the paper point content was re-eluted in the tubes with 300 µl of sterile phosphate buffered saline (PBS), containing a protease inhibitor (complete mini EDTA free, Roche, Basel, Switzerland). The tubes were placed for 5 h on a platform shaker at (2000 rpm) at 4°C, vortexed for 30 sec, and thereafter centrifuged for 10 min at 5000 rpm. The resulting cell pellet was collected and processed for FISH analysis, as described further.

## ***2.3 Analysis of apical samples by fluorescent in situ hybridization (FISH) and epifluorescence microscopy***

The detection and counting of bacteria in the prepared samples was performed by FISH, followed by epifluorescence microscopy, in similar principles as previously described [8]. Briefly, 50 µl of 0.9% NaCl containing RNase inhibitor (Sigma Aldrich, Buchs, Switzerland) were added onto the pelleted sample, followed by shaking for 45 min, and vortexing for 1 min. Then, 10 µl of the suspensions were mixed with 5 µl of coating buffer (0.9% NaCl, 0.02% NaN<sub>3</sub>, 2.5 x 10<sup>-4</sup>% hexadecyltrimethylammonium bromide) on multi-well epoxy coated Adcell slides, with a well-diameter of 4 mm (Cel-Line, Erie Scientific Company, Portsmouth, NH, USA). The slides were air dried and fixed by a 20 min-incubation in 4% paraformaldehyde at 4°C, washed with nanopure H<sub>2</sub>O and then processed for FISH analysis [8]. Every well was covered with 9 µl Denhardt's Solution (diluted 1:50 in PBS), including 1:500 RNase inhibitor, to reduce non-specific probe binding to the

bacterial cell wall. The slides were then incubated for 30 min at 37°C. Four specific oligonucleotide rRNA probes were used for *Synergistetes* cluster A and *Synergistetes* cluster B bacteria [6, 8], a genus-specific probe for oral *Fusobacteria* [18], and a genus-specific probe for oral *Actinomyces* [19]. The cluster classification of oral *Synergistetes* bacteria into A and B was based on earlier studies [2, 6, 8]. Table 1 lists the oligonucleotide sequences and Cy3 or 6-FAM labeling of the used rRNA probes (Microsynth, Balgach, Switzerland), as well as their targeted taxa. The final probe concentrations used for FISH were 5 ng/μl for Cy3 conjugates and 20 ng/μl for FAM conjugates, in the presence of 40% formamide. For hybridization, 3-4 μl of probe solution was added to the wells, and incubated for 4 h, at 46°C. Thereafter, they were washed for 30 min, air-dried and covered with 50 μl mounting fluid and a cover-slip. An Olympus BX60 fluorescence microscope (Olympus Optical AG, Volketswil, Switzerland) was used for the quantitative evaluation of the FISH stained samples. Fluorescence and direct light images of the detected bacteria were taken by an Olympus E510 camera. The quantitative evaluation of the stained bacterial taxa was done by counting the fluorescent bacterial cells in at least ten viewing fields per well, at 100x magnification, as previously described [20]. The lowest detection limit of the assay was 50 bacterial counts per sample.

## **2.4 Statistical analysis**

The Chi-square test was used to compare the frequencies of detection of the different bacterial taxa among the three clinical diagnosis groups. Statistical significance level was set at  $P < 0.05$ .



### 3. Results

The presence of *Synergistetes* in the apical samples obtained from the three different clinical conditions was evaluated using FISH and epifluorescence microscopy. The frequencies of detection of the different taxa were first calculated (Table 2). It was found that *Synergistetes* cluster A bacteria were present 10% of N-AP cases, in 24% of R-AP cases, but none of pulpitis cases. Interestingly, *Synergistetes* cluster B was not detectable in any of the groups. To confirm the presence of well-established endodontic pathogens in apical periodontitis, the presence of *Actinomyces* and *Fusobacterium* genera were further investigated using 16S rRNA genera-specific oligonucleotide probes. It was found that *Actinomyces* were detected in 12% of N-AP cases, 48% of R-AP cases, but none of the pulpitis cases. On the other hand *Fusobacteria* were detected in 12% of N-AP cases, 38% of R-AP cases, and 11% of the pulpitis cases. These differences in detection proved to be statistically significant between clinical diagnosis groups for all taxa analysed, with the exception of *Synergistetes* cluster B, which was undetectable under the present conditions. Regarding the numerical levels of detection of the investigated taxa, these ranged between 50 – 255 counts for *Synergistetes* cluster A bacteria, 70 – 1650 counts for *Actinomyces*, and 50 – 720 counts for *Fusobacteria* (Table 3). The highest bacterial numbers were detected in samples with clinical diagnosis of R-AP, followed by N-AP.

The investigated bacterial taxa were further characterized morphologically, as appeared by FISH staining under epifluorescence microscopy. The *Synergistetes* cluster A bacteria, detected only in the N-AP and R-AP cases, appeared as long rods, which were either in straight form (Figure 1A, B) or curved form (Figure 1C, D). *Fusobacteria* appeared as elongated spindle-shaped rods either as single cell bodies (Figure 2A-D) or forming complexes of several bacterial cells (Figure 2E-H).

*Actinomyces* appeared as irregularly shaped long rods (Figure 3A-D), occasionally forming intertwining filamentous structures (Figure 3E, F).

#### 4. Discussion

In summary, the presence and levels of *Synergistetes* was evaluated in apical samples of teeth diagnosed with N-AP or R-AP. As clinical control, a pulpitis group was employed, whereas as microbiological controls, the presence of well-established endodontic pathogens (i.e. *Fusobacteria* and *Actinomyces*) was evaluated. Sampling from the root canal system is not free from methodological pitfalls [21]. One problem with root canal sampling, as with periodontal sampling, is the fact that microorganisms from sites other than the front of the lesion are collected along with counterparts that cause disease progression. In the current study, it was aimed to sample from the apical aspect of the root canal system, and thus, NaOCl was used to lyse the bacteria that may have been present in the coronal aspects of the root canal. However, as can be seen by the fact that *Fusobacteria* could be sampled in 11% of the irreversible pulpitis cases, which should essentially be bacteria-free in the apical region [11], we cannot necessarily claim that the current sampling procedure predictably prevented false-positive results in the sense that bacteria were transported from the tooth crown to the apex during root canal preparation prior to sampling. Nevertheless, the current results clearly show that there is a vast difference in numbers of recovered taxa between teeth with apical periodontitis and counterparts with irreversible pulpitis.

It was found that *Synergistetes* cluster A bacteria were detected in both N-AP and R-AP but not in pulpitis. Their prevalence as well as detection range was greater in R-AP compared to N-AP. *Synergistetes* cluster B was not detected in any of the

three clinical diagnosis groups, implying that it is not crucial in the establishment of AP, as has also been demonstrated in the case of marginal periodontal diseases [6, 8]. Earlier works using other molecular assays to detect *Synergistetes* in samples obtained from the apical part of N-AP or R-AP-affected teeth and analyzed by 16S rRNA-based nested or hemi-nested PCR, or by reverse-capture checkerboard hybridization assays. In these studies, the main *Synergistetes* oral clones detected included BA121 (now designated as *Pyramidobacter piscolens*), BH017/D084, W090 (now designated as *Fretibacterium fastidiosum*) and E3\_33 (now designated as *Jonquetella anthropi*), and were found at prevalence of up-to 33%, [16, 17, 22, 23]. Among those, *Synergistes* oral clone BA121 (*Pyramidobacter piscolens*) was the most frequently detected (29%) [23, 24], at levels exceeding  $10^5$  DNA counts [25], and was also among the most frequently detected taxa in endodontic abscesses [26]. Hence, the prevalence reported in these earlier studies using PCR-based methods is well in agreement findings of the present study for the prevalence of *Synergistetes* cluster A in N-AP and R-AP (10% and 24%, respectively). Yet, some of the previously identified species (*Pyramidobacter piscolens* and *Jonquetella anthropi*) belong to *Synergistetes* cluster B, while the present study failed to detect bacteria in this cluster. The possible explanation for this discrepancy is that the cluster B species described to date are smaller in size than cluster A taxa, so they may be harder to detect by FISH. Moreover, the 16S rRNA probe used in the present study was designed for a broad range of cluster B clones, and not exclusively for these two species [8]. This study additionally demonstrates the morphology *Synergistetes* cluster A in apical samples, and confirms the absence of any *Synergistetes* in pulpitis. The structure of *Synergistetes* cluster A described here by FISH, resembles that of *Synergistetes* identified in dental plaque of gingivitis patients, using the same method [8].

Bacteria of the genus *Actinomyces* are highly prevalent in the polymicrobial communities of infected dental root canals [27], and account for the majority of Gram-positive rods identified in AP [28]. Their persistence in the root canal is associated with failed endodontic treatment and incomplete periapical healing, as identified by bacterial cultivation [29], or by PCR-based methods [30]. The high prevalence of *Actinomyces* and *Fusobacteria* in the apical region of teeth with endodontic infection has also been demonstrated by pyrosequencing analysis, which revealed great diversity in the bacterial communities of the affected teeth, as well as a great inter-individual diversity [31]. Accordingly, when the bacterial communities of the apical portion of N-AP or R-AP-affected teeth were compared, a high prevalence of *Fusobacteria* and *Actinomyces* was confirmed, with R-AP displaying a greater diversity in bacterial composition [32]. Peri-radicular lesion samples analyzed by a 454-sequencing platform revealed that among the most abundant genera, as represented by the total yield of sequences, were *Fusobacterium* and *Actinomyces* [33]. In the present study, *Actinomyces* and *Fusobacteria* were detected in both N-AP and R-AP, albeit at higher prevalence and numeric range in the latter case. These findings are well in line with the literature. The novelty of the present study is the confirmation of the presence of *Actinomyces* and *Fusobacteria* in periapical pathoses by FISH staining and microscopic visualization. With the help of the selected probes used, the bacteria could be identified the genus level, but not the phylotype level. The fact that these bacteria have been microscopically identified here proves their apical presence by culture-independent methods. However, this does not necessarily imply that they would be retrievable by cultivation, as their density was relatively low. Nevertheless, the method provides the possibility for descriptive information on the morphology of the microorganisms under investigation. Indirect immunofluorescence

microscopy has been used earlier to determine the presence of selected *Actinomyces* species in endodontic infections. These were identified in 60% of the cases [34], which is proximal to the 48% prevalence in R-AP, reported in this study.

In conclusion, the findings of this study represent a snapshot of the microbial complexity in the apical root canal region. The FISH method employed here allows for the visual identification and relative enumeration of the targeted taxa, irrespective of their cultivability. The physical presence of *Synergistetes* cluster A bacteria was confirmed in both N-AP and R-AP, whereas this was absent in pulpitis. A stronger presence of this cluster was evident in R-AP, while cluster B was non-detectable in any of clinical diagnosis groups. In conclusion, *Synergistetes* cluster A, but not cluster B, bacteria can be found intact as part of the mixed apical microbiota of AP infections, and may be involved in their pathogenesis. Whether this association represents a causal relationship with the disease, or is merely a result of opportunistic infection remains to be elucidated.

## Acknowledgements

The authors would like to thank Mrs Helga Lüthi-Schaller for her excellent technical support. The study was funded by the authors' Institute.

## References

- [1] Horz HP, Citron DM, Warren YA, Goldstein EJ, Conrads G. Synergistes group organisms of human origin. *Journal of clinical microbiology*. 2006;44:2914-20.
- [2] Vartoukian SR, Palmer RM, Wade WG. Diversity and morphology of members of the phylum "synergistetes" in periodontal health and disease. *Applied and environmental microbiology*. 2009;75:3777-86.

- [3] Vartoukian SR, Palmer RM, Wade WG. The division "Synergistes". *Anaerobe*. 2007;13:99-106.
- [4] Marchandin H, Damay A, Roudiere L, Teyssier C, Zorziotti I, Dechaud H, et al. Phylogeny, diversity and host specialization in the phylum Synergistetes with emphasis on strains and clones of human origin. *Research in microbiology*. 2010;161:91-100.
- [5] Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. *Journal of bacteriology*. 2010;192:5002-17.
- [6] Belibasakis GN, Ozturk VO, Emingil G, Bostanci N. Synergistetes cluster A in saliva is associated with periodontitis. *Journal of periodontal research*. 2013;48:727-32.
- [7] You M, Mo S, Watt RM, Leung WK. Prevalence and diversity of Synergistetes taxa in periodontal health and disease. *Journal of periodontal research*. 2013;48:159-68.
- [8] Baumgartner A, Thurnheer T, Luthi-Schaller H, Gmur R, Belibasakis GN. The phylum Synergistetes in gingivitis and necrotizing ulcerative gingivitis. *J Med Microbiol*. 2012;61:1600-9.
- [9] Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology*. 2003;149:279-94.
- [10] Bjorndal L, Reit C. The annual frequency of root fillings, tooth extractions and pulp-related procedures in Danish adults during 1977-2003. *International endodontic journal*. 2004;37:782-8.
- [11] Langeland K. Tissue response to dental caries. *Endodontics & dental traumatology*. 1987;3:149-71.

- [12] Hahn CL, Liewehr FR. Innate immune responses of the dental pulp to caries. *Journal of endodontics*. 2007;33:643-51.
- [13] Gusman H, Santana RB, Zehnder M. Matrix metalloproteinase levels and gelatinolytic activity in clinically healthy and inflamed human dental pulps. *European journal of oral sciences*. 2002;110:353-7.
- [14] Nair PN. Apical periodontitis: a dynamic encounter between root canal infection and host response. *Periodontology 2000*. 1997;13:121-48.
- [15] Machado de Oliveira JC, Gama TG, Siqueira JF, Jr., Rocas IN, Peixoto RS, Rosado AS. On the use of denaturing gradient gel electrophoresis approach for bacterial identification in endodontic infections. *Clinical oral investigations*. 2007;11:127-32.
- [16] Siqueira JF, Jr., Rocas IN. Molecular detection and identification of *Synergistes* phylotypes in primary endodontic infections. *Oral diseases*. 2007;13:398-401.
- [17] Vianna ME, Conrads G, Gomes BP, Horz HP. Quantification and characterization of *Synergistes* in endodontic infections. *Oral microbiology and immunology*. 2007;22:260-5.
- [18] Thurnheer T, Gmur R, Guggenheim B. Multiplex FISH analysis of a six-species bacterial biofilm. *Journal of microbiological methods*. 2004;56:37-47.
- [19] Guggenheim B, Gmur R, Galicia JC, Stathopoulou PG, Benakanakere MR, Meier A, et al. In vitro modeling of host-parasite interactions: the 'subgingival' biofilm challenge of primary human epithelial cells. *BMC microbiology*. 2009;9:280.
- [20] Gmur R, Thurnheer T. Direct quantitative differentiation between *Prevotella intermedia* and *Prevotella nigrescens* in clinical specimens. *Microbiology*. 2002;148:1379-87.

- [21] Sathorn C, Parashos P, Messer HH. How useful is root canal culturing in predicting treatment outcome? *Journal of endodontics*. 2007;33:220-5.
- [22] Rocas IN, Baumgartner JC, Xia T, Siqueira JF, Jr. Prevalence of selected bacterial named species and uncultivated phylotypes in endodontic abscesses from two geographic locations. *Journal of endodontics*. 2006;32:1135-8.
- [23] Siqueira JF, Jr., Rocas IN. Uncultivated phylotypes and newly named species associated with primary and persistent endodontic infections. *Journal of clinical microbiology*. 2005;43:3314-9.
- [24] Rocas IN, Hulsmann M, Siqueira JF, Jr. Microorganisms in root canal-treated teeth from a German population. *Journal of endodontics*. 2008;34:926-31.
- [25] Siqueira JF, Jr., Rocas IN, Alves FR, Silva MG. Bacteria in the apical root canal of teeth with primary apical periodontitis. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics*. 2009;107:721-6.
- [26] Siqueira JF, Jr., Rocas IN. The microbiota of acute apical abscesses. *Journal of dental research*. 2009;88:61-5.
- [27] Borssen E, Sundqvist G. Actinomyces of infected dental root canals. *Oral surgery, oral medicine, and oral pathology*. 1981;51:643-8.
- [28] Chavez de Paz LE, Molander A, Dahlen G. Gram-positive rods prevailing in teeth with apical periodontitis undergoing root canal treatment. *International endodontic journal*. 2004;37:579-87.
- [29] Sjogren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *International endodontic journal*. 1997;30:297-306.
- [30] Xia T, Baumgartner JC. Occurrence of Actinomyces in infections of endodontic origin. *Journal of endodontics*. 2003;29:549-52.



- [31] Rocas IN, Siqueira JF, Jr. Comparison of the in vivo antimicrobial effectiveness of sodium hypochlorite and chlorhexidine used as root canal irrigants: a molecular microbiology study. *Journal of endodontics*. 2011;37:143-50.
- [32] Chugal N, Wang JK, Wang R, He X, Kang M, Li J, et al. Molecular characterization of the microbial flora residing at the apical portion of infected root canals of human teeth. *Journal of endodontics*. 2011;37:1359-64.
- [33] Saber MH, Schwarzberg K, Alonaizan FA, Kelley ST, Sedghizadeh PP, Furlan M, et al. Bacterial flora of dental periradicular lesions analyzed by the 454-pyrosequencing technology. *Journal of endodontics*. 2012;38:1484-8.
- [34] Gohean RJ, Pantera EA, Jr., Schuster GS. Indirect immunofluorescence microscopy for the identification of *Actinomyces* sp. in endodontic disease. *Journal of endodontics*. 1990;16:318-22.

## Tables

**Table 1.** 16S rRNA-targeted probe sequences for FISH and target taxa

Probe	Sequence (5'–3')	Target taxa
SYN-A1409-FAM	ACACCCGGCTCGGGTGGT	<i>Synergistetes</i> cluster A
SYN-B1149-Cy3	TCGATGGCAGTCTCGCCG	<i>Synergistetes</i> cluster B
L-ACT476-2-FAM	ATCCAGCTACCGTCAACC	genus <i>Actinomyces</i>
FUS664-Cy3	CTTGTAGTTCCGCTACCTC	genus <i>Fusobacterium</i>

**Table 2.** Frequency of detection of different taxa in apical samples per clinical diagnosis.

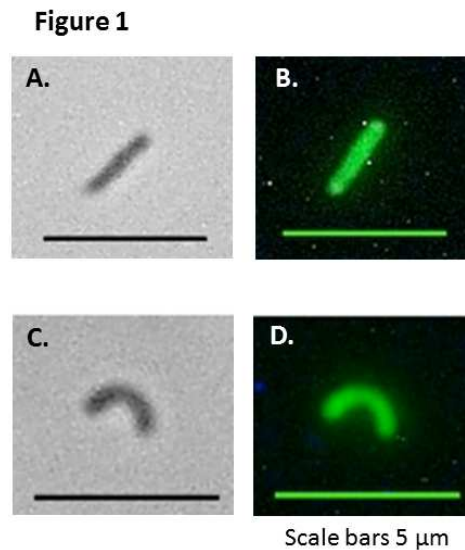
	Pulpitis	N-AP	R-AP	P value
<i>Synergistetes</i> cluster A	0/27 (0%)	3/33 (10%)	5/21 (24%)	0.022

<i>Synergistetes</i> cluster B	0/27 (0%)	0/33 (0%)	0/21 (0%)	N/A
<i>Actinomyces</i>	0/27 (0%)	4/33 (12%)	10/21 (48%)	<0.0001
<i>Fusobacteria</i>	3/27 (11%)	4/33 (12%)	8/21 (38%)	0.027

**Table 3.** Range of detection (bacterial counts) of different taxa in the apical samples per clinical diagnosis.

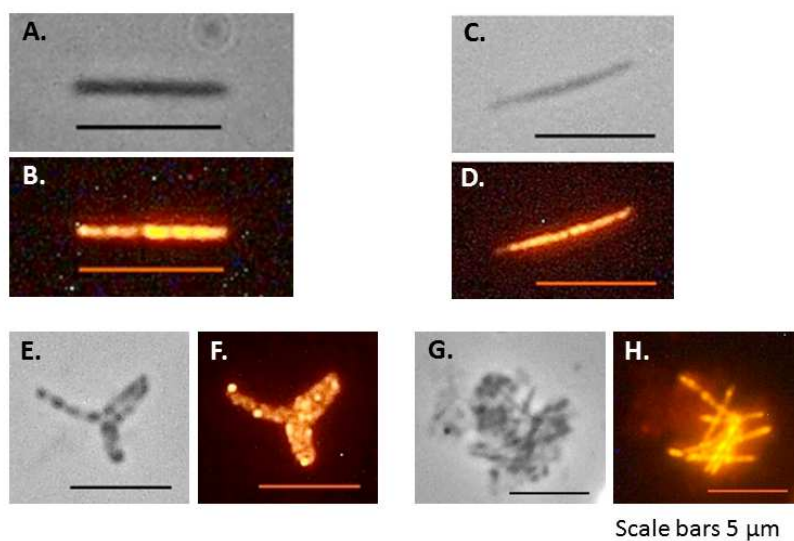
	<b>Pulpitis</b>	<b>N-AP</b>	<b>R-AP</b>
<i>Synergistetes</i> cluster A	not detected	50 – 90	70 – 255
<i>Synergistetes</i> cluster B	not detected	not detected	not detected
<i>Actinomyces</i>	not detected	70 – 600	55 – 1650
<i>Fusobacteria</i>	50 – 105	70 – 135	50 – 720

## Figures



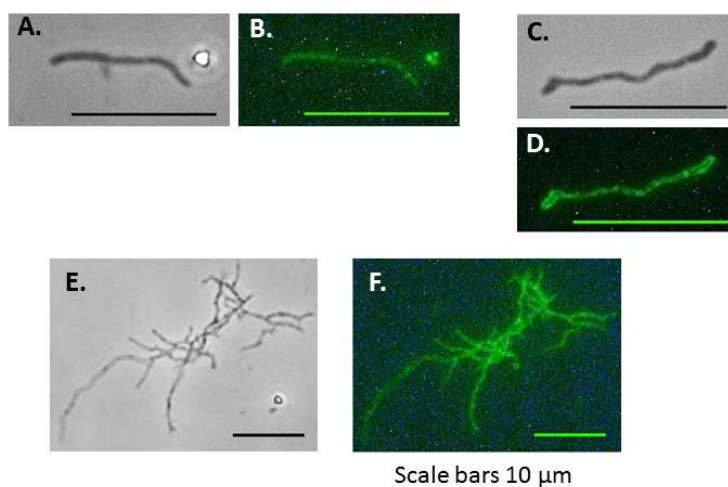
**Figure 1.** Morphology of bacteria from *Synergistetes* cluster A in apical samples, as identified by FISH. (A and C) Phase contrast and (B and D) corresponding epifluorescence images stained with SYN-A1409-FAM probe. Scale bar = 5 μm.

**Figure 2**



**Figure 2.** Morphology of *Fusobacteria* bacteria in apical samples, as identified by FISH. (A, C, E, and G) Phase contrast and (B, D, F and H) corresponding epifluorescence images stained with FUS664-Cy3 probe. Scale bar = 5 μm.

**Figure 3**



**Figure 3.** Morphology of *Actinomyces* bacteria in apical samples, as identified by FISH. (A, C and E) Phase contrast and (B, D and F) corresponding epifluorescence images stained with L-ACT476-2-FAM probe. Scale bar = 10 μm.